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Award Number: DAMD17-01-1-0090

TITLE: Targeted Delivery of Therapeutic Oligonucleotides for the Treatment of Prostate Cancer

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REPORT DATE: May 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual (1 May 2002 - 30 Apr 2003)

4. TITLE AND SUBTITLE
Targeted Delivery of Therapeutic Oligonucleotides for the Treatment of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0090

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Down-regulation of Bcl-2 expression via antisense oligodeoxynucleotides (ODN) may show promise as a novel therapy for the treatment of prostate cancer. The success of the antisense therapy is largely dependent on the development of a vector that is highly efficient in selective delivery of ODN to prostate cancer cells. To this end we have developed a novel lipid vector that is highly efficient in encapsulating ODN. Using folate as a model ligand we have shown that incorporation of folate into the lipid vector resulted in a significant improvement in intracellular delivery of ODN to KB cells that overexpress folate receptors. Targeted delivery of an EGFR antisense ODN via the novel lipid vector led to a dramatic reduction in the EGFR expression in KB cells. In a separate study we have shown that a small molecule glutamate carboxypeptidase inhibitor (DβE) efficiently mediates liposomal targeting to LNCaP prostate cancer cells that overexpress prostate specific membrane antigen (PSMA). These studies pave the way for the future development of PSMA-specific lipid vectors to selectively deliver Bcl-2 antisense ODN to prostate cancer cells.

14. SUBJECT TERMS
Targeted therapy; Drug delivery system; Antisense; Prostate cancer;

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

Androgen independence and chemoresistance are the major obstacles in the treatment of patients with advanced prostate cancer (Denis & Murphy, 1993; Oh & Kantoff, 1998). Recent studies have suggested that overexpression of a proto-oncorprotein, Bcl-2, plays an important role in the development of androgen independence and chemoresistance in prostate cancer (McDonnell et al., 1992; Colombel et al., 1993; Berchem et al., 1995; Raffo et al., 1995; Bauer et al., 1996, McConkey et al., 1996). We propose in this application to develop a tumor-specific vehicle to selectively deliver to the tumor an antisense oligodeoxynucleotides (ODN) that is targeted to Bcl-2. Down-regulation of Bcl-2 in prostate cancer could render it more susceptible to the standard chemotherapy. Sigma receptor will be chosen as the target as they are overexpressed in a number of tumors including prostate cancer (Vilner et al., 1995; John et al., 1993; John et al., 1995a; John et al., 1995b; John et al., 1996; John et al., 1999a; John et al., 1999b).

BODY:

Two specific aims were originally proposed in our application and a three-year support was requested. A two-year support was later approved to request us to focus on the first aim: ***to develop novel delivery systems for targeted delivery of ODN to prostate cancer***. Two issues are critical for achieving this goal: 1) to identify a targeting ligand that is highly specific for prostate cancer; and 2) to develop a lipid vector that is highly efficient in encapsulating ODN and in mediating selective delivery of ODN to target cells. We have shown in previous report that anisamide derivatives efficiently mediated delivery of liposomal doxorubicin (DOX) to DU-145 prostate cancer cells that overexpress sigma receptors. Since then we have developed another liposomal targeting liagnd (DβE) that is highly specific for prostate specific membrane antigen (PSMA). Furthermore we have developed a new lipid vector that is highly efficient in mediating intracellular delivery of ODN to target cells in vitro and in vivo.

1. Targeted delivery of liposomal drugs to prostate cancer cells. In collaboration with Dr. Leaf Huang, we have recently shown that anisamide-derivatized ligand efficiently mediates delivery of liposomal doxorubicin (DOX) to DU-145 prostate cancer cells that overexpress sigma receptor in vitro and in vivo (Banerjee, R., Li, S., & Huang, L., *Cancer Research, in revision*). Targeted DOX delivery not only improved the antitumor activity but also decreased the DOX-related toxicity (Banerjee, R., Li, S., & Huang, L., *Cancer Research, in revision*). More recently we have shown that a small molecule glutamate carboxypeptidase inhibitor (DβE) also efficiently mediates liposomal targeting to LNCaP cells that overexpress PSMA. Figure 1 shows the uptake of the rhodamine (Rho)-labeled liposomes 1 h following incubation with LNCaP cells at 37°C. DβE-conjugated liposomes were more efficiently taken up by LNCaP cells than the control non-targeted liposomes. Liposomal targeting appears to be specifically mediated by PSMA because there is only a background level of liposomal uptake in DU-145 cells that lack the expression of PSMA (data not shown). We are currently examining whether DβE can be employed for targeted delivery of liposomal drugs (including ODN) to PSMA-

overexpressing prostate cancer cells. PSMA-mediated targeting might be advantageous over sigma receptor-based targeting since PSMA is more specific for prostrate cancer. Several studies have shown that PSMA is also overexpressed in what appears to be microvascular lining cells in prostate cancer and other tumors (Liu et al., 1997; Liu et al., 2002). Thus there might be a synergistic effect in PSMA-mediated targeting by killing both tumor cells and tumor endothelial cells.

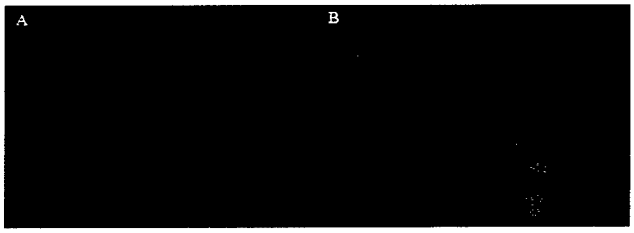

Fig. 1. DβE-mediated liposomal targeting to LNCaP cancer cells. *Rho-labeled liposomes composed of PC, cholesterol and PE-PEG with (B) or without (A) DSPE-PEG-DβE (0.5 mol%) were prepared and added to LNCaP cells. Liposomal uptake was examined one h later under a fluorescence microscope.*

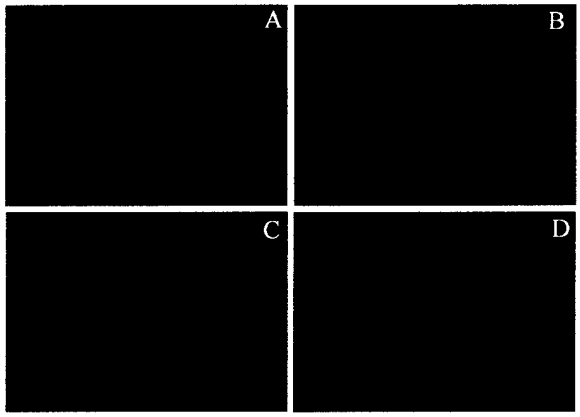

Fig. 2. EGFR expression in KB cells following targeted delivery of EGFR antisense ODN. *Cells were treated with ODN, free or formulated in FR-targeted lipid vesicles for 1 h and then cultured in normal medium. Expression of EGFR in KB cells 4 days following ODN treatment was then examined by an indirect immunofluorescent staining using mouse anti-human EGFR antibody. A: KB cells without treatment; B: negative control (cells reacted with blocking buffer followed by fluorescein-labeled goat anti-mouse IgG); C: KB cells treated with free EGFR antisense ODN; D: KB cells treated with antisense ODN formulated in FR-targeted lipid vesicles.*

2. Development of a novel lipid vector for targeted delivery of ODN.

The major limitation with liposomal vectors for ODN delivery is the low encapsulation efficiency. A novel method has recently been reported to encapsulate ODN inside lipidic vesicles at a high efficiency (Semple et al., 2001). In this study, we were examining whether this vector can be modified to achieve targeted ODN delivery. This feasibility was examined using folate as a model ligand. A number of studies have shown that certain types of tumors overexpress folate receptors (FR). Folate has been used by our group and other groups to target anticancer agents to tumors that overexpress FR. Conventional FR-targeted liposomes suffer from a low entrapment efficiency for ODN delivery. We have successfully prepared FR-targeted lipidic ODN with ODN entrapment efficiency as high as 60-80% (w/w). The lipid composition is composed of DSPC: cholesterol: DODAP: DSPE-PEG: folate-PEG-PE. The

folate mediates efficient targeting of ODN to KB cells that overexpress FR (Zhou et al., 2002, included as appendix 1). Targeted delivery of EGFR antisense ODN to KB cells led to a dramatic reduction in the EGFR expression as shown in an immuno-fluorescence assay (Figure 2), far more efficient than free antisense ODN. Down-regulation of EGFR expression via targeted antisense therapy greatly sensitizes KB cells to chemotherapy. Figure 3 shows the results of a MTT assay following treatment of KB cells with FR-targeted EGFR antisense ODN, doxorubicin (Dox), or a combination of both. Either antisense ODN or Dox exhibited only a low level of cytotoxic effect on KB cells. However, pretreatment with FR-targeted EGFR antisense ODN significantly enhances the cytotoxic effect of Dox on KB cells. We are currently extending this study to targeted delivery of ODN to prostate cancer via PSMA.

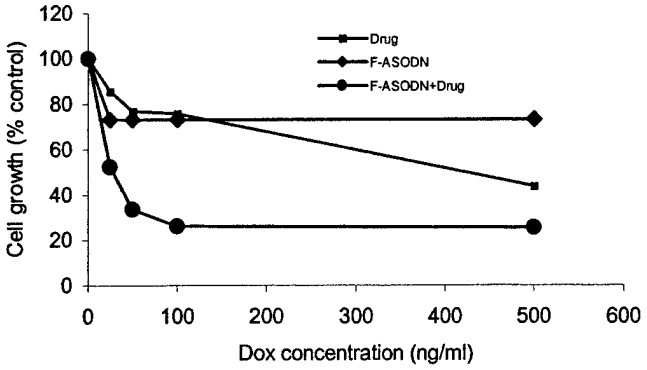

Fig. 3. Treatment with FR-targeted antisense ODN sensitizes KB cells to chemotherapy. *KB cells were treated with FR-targeted antisense ODN for 1 h and then cultured in normal medium. Two days later cells were exposed to various concentrations of Dox and cell growth was examined 1 day later by MTT assay.*

KEY RESEARCH ACCOMPLISHMENTS:

We have demonstrated for the first time that small molecular weight ligands including benzamide derivatives and DβE can be used to target liposomal drugs to prostate cancer cells in vitro and in vivo. The advantages of these targeting systems include: a) low immunogenecity; b) low toxicity due to the excellent safety profiles of benzamide and DβE. This strategy might be used to deliver to the prostate cancer cells various types of anticancer agents including small molecular weight chemotherapeutic drugs, ODN and genes. Using folate as a model, we also developed a method to prepare targeted liposomal ODN with a high ODN entrapment efficiency. The success of these studies will pave the way to our proposed project of targeted delivery of Bcl-2 antisense ODN to prostate cancer.

REPORTABLE OUTCOMES:

Zhou, W., Yuan, X., Wilson, A., Yang, L., Mokotoff, M., Pitt, B., and Li, S. Efficient intracellular delivery of oligonucleotides formulated in folate receptor-targeted lipid vesicles. *Bioconjuagte Chemistry* 13: 1220-1225, 2002.

Banerjee R, Li, S., and Huang, L. Anisamide-targeted stealth liposomes: a potent carrier for targeting doxorubicin to prostate cancer cells. *Cancer Research* (in revision)

Yang, L., Zhou, W., and Li, S. Targeted delivery of antisense oligonucleotides to folate receptor-overexpressing tumor cells. Submitted to *Molecular Cancer Therapy*

Zhou, W., Yuan, X., Yang, L., Gao, X., and Li, S. Prostate-specific membrane antigen-mediated liposomal targeting to prostate cancer. (in preparation)

CONCLUSIONS:

Sigma receptors or PSMA mediate efficient targeting of liposomal drugs to prostate cancer. We are currently extending this study to targeting of Bcl-2 antisense oligonucleotides to prostate cancers.

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Efficient Intracellular Delivery of Oligonucleotides Formulated in Folate Receptor-Targeted Lipid Vesicles

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In this study, a novel lipid vector has been developed for targeted delivery of oligodeoxynucleotides (ODN) to tumors that overexpress folate receptor. This is based on a method developed by Semple et al. (*1*), which utilizes an ionizable aminolipid (1,2-dioleoyl-3-(dimethylammonio)propane, DODAP) and an ethanol-containing buffer system for encapsulating large quantities of polyanionic ODN in lipid vesicles. Folate is incorporated into the lipid vesicles via a distearoylphosphatidylethanolamine poly-(ethylene glycol) (DSPE PEG) spacer. These vesicles are around 100 200 nm in diameter with an ODN entrapment efficiency of 60 80%. Folate mediated efficient delivery of ODN to KB cells that overexpress folate receptor. Uptake of folate-targeted lipidic ODN by KB cells is about 8 10-fold more efficient than that of lipidic ODN without a ligand or free ODN. This formulation is resistant to serum. Thus, targeted delivery of ODN via this novel lipid vector may have potential in treating tumors that overexpress folate receptors.

INTRODUCTION

Functional oligodeoxynucleotides (ODN) such as antisense ODN and DNA enzymes hold promise as new therapeutics for the treatment of various types of diseases such as cancers (*2*, *3*). A number of studies have suggested the existence of ODN receptors on the cell surface (*4*, *5*). However, intracellular delivery of ODN without a delivery vehicle is generally inefficient. Successful application of therapeutic ODN is largely dependent on the development of a vehicle that selectively delivers the ODN into target cells with minimal toxicity. A number of vectors have been developed to improve the intracellular delivery of ODN such as neutral liposomes (*6*) and cationic liposomes (*7*, *8*). ODN can be entrapped inside neutral liposomes. The liposomes can also be designed such that they are long-circulating in the blood and target cell-specific. However, the size of liposomes required for achieving long circulation in the blood and efficient localization is too small for efficient entrapment of ODN. Cationic liposomes readily form complexes with ODN through electrostatic interactions. Almost 100% of ODN can be recovered in complex form. The resulting complexes usually contain slight excess amount of positive charges that allows efficient interaction with the negatively charged cell membrane. A number of cationic liposomes have been developed that significantly enhance the intracellular delivery of ODN. ODN complexed with cationic liposomes exhibit biological activity up to 1000-fold higher than ODN alone (*7*, *8*). However, targeted delivery of ODN to solid tumors via systemic administration remains problematic, although we have shown that cationic liposomes mediate efficient delivery of ODN to pulmonary endothelium (*9*). Recently, a novel lipid formulation has been developed (*1*) that appears to avoid the problems that are associated with each of the formulations discussed above. This formulation is composed of distearoylphosphatidylcholine (DSPC), cholesterol, DODAP, and *N*-palmitoylsphingosine-1-[succinyl-(methoxypoly(ethylene glycol))2000] (PEG-CerC_{16}). DODAP is an ionizable cationic lipid that has a pK of 6.6 in lipid bilayer systems. At acidic pH values (i.e. pH 4.0), this lipid is positively charged and helps to improve the ODN entrapment via electrostatic interactions. DODAP/ODN complexes can interact with other lipids in an ethanol-containing buffer, and ODN-containing lipid vesicles are formed upon the removal of the ethanol by dialysis. Subsequent adjustment of the external pH to neutral pH values results in a neutral surface charge on the resulting particles. This method led to a significant increase in ODN entrapment efficiency (60 80%) with a final lipid/ODN ratio of 0.15 0.25 (w/w) (*1*). This formulation has also been shown to be long circulating in the blood following systemic administration (*1*). In this study, we have shown that this formulation can be further improved via the incorporation of a targeting ligand, folate. Folate-targeted vesicles are much more efficient than the nontargeted vectors in delivering ODN to KB cells. This improved formulation may have potential for the targeted delivery of therapeutic oligonucleotides to tumors that overexpress folate receptors.

EXPERIMENTAL PROCEDURES

Lipids and Chemicals. Distearoylphosphatidylcholine (DSPC), *N*-palmitoylsphingosine-1-[succinyl(methoxypoly(ethylene glycol))2000] (PEG-CerC_{16}), and 1,2-dioleoyl-3-(dimethylammonio)propane (DODAP) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Folic acid

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10.1021/bc025569z CCC: $22.00
Published on Web 10/23/2002

Table 1. The Sequences of ODN Used in This Study

no.	sequence (5′-3′)
1	tat gat ctg tca cag ctt ga (*19*)
2	cgg agg gtc gca tcg ctg (*20*)
3	cac gcc ctt acc ttt ctt ttc ct (*20*)
4	ccg tgg tca tgc tcc (*6*)
5	ccc cag cag ctc cca ttg gg (*21*)
6	gct gac gca ctg act (*6*)

and cholesterol (CH) were purchased from Sigma-Aldrich. All lipids were 99% pure. Folate-free RPMI 1640 medium, F12 medium, and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). Folate poly(ethylene glycol) (MW ~ 3350 or 2000 Da) distearoyl phosphatidylethanolamine (folate PEG DSPE) was synthesized as described previously (10). The final folate PEG DSPE product was purified on a column of silica gel (70 200 mesh) using a solvent gradient of 15 to 80% methanol in CH_2Cl_2. Product purity was confirmed by thin-layer chromatography analysis on silica gel GF.

Antisense ODN. All ODN were synthesized with phosphorothioate (PS) backbone chemistry (the Midland Certified Reagent Company, Midland, TX). The sequences of ODN used in this study are shown in Table 1.

Preparation of ODN-Containing Lipid Vesicles. ODN-containing lipid vesicles were prepared according to the method described by Semple et al. (*1*) except that folate PEG DSPE was added when preparing the folate-targeted lipid vesicles. Briefly, a lipid solution composed of DSPC, cholesterol, DODAP, and PEG-CerC_{16} in 100% ethanol at indicated molar ratios (Figures 1 7) was prepared. For preparation of folate-targeted lipid vesicles, various amounts of folate PEG DSPE were added. To the lipid solution was then added 300 mM citric acid, pH 4.0 to a final ethanol concentration of 40% (v/v). Similarly, ODN were prepared in separate tubes in 300 mM citric acid, pH 4.0, with 40% ethanol. The solutions were prewarmed to 65 °C, and then the lipid solution was slowly added to the ODN with gentle vortexing. The input ratio was 150 μg ODN/μmol lipid. The mixture was passed 10 times through three stacked 100 nm polycarbonate filters. The preparation was dialyzed (12 14 kDa cutoff) against 300 mM citrate buffer, pH 4.0, for approximately 1 h to remove excess ethanol and further dialyzed against HBS (20 mM HEPES, 145 mM NaCl, pH 7.6) for 12 18 h to remove the citrate buffer, neutralize the DODAP, and release any ODN that was associated with the surface of the vesicles. ODN-containing lipid vesicles were finally separated from free ODN via gel filtration on a Sepharose CL-4B column (1 cm × 25 cm).

Cells and Medium. KB human cancer cells, derived from an epidermal carcinoma of the oral cavity, were obtained from American Type Culture Collection (ATCC). The cells were maintained in folate-free RPMI 1640 medium supplemented with 100 units/mL penicillin, 100 g/mL streptomycin, and 10% fetal bovine serum, which provides the only source of folate (the final folate concentration in the serum-supplemented medium was approximately physiological). The cells were cultured as a monolayer in a humidified atmosphere containing 5% CO_2 at 37 °C. CHO cells were obtained from ATCC and cultured in F12 medium supplemented with 10% fetal calf serum.

Uptake of ODN by KB and CHO Cells. Cells were seeded to a 24-well plate at a cell density of 2.5×10^4/well and allowed to grow overnight. ODN, free or encapsulated in lipid vesicles, were added to the cells in various concentrations [determined according to a carbocyanine dye (Cy3)-labeled ODN]. Following incubation at 37 °C for 1 h, cells were washed with PBS (3 × 3 min). Cells were lysed using a lysis buffer (0.1% Triton X in 100 mM Tris, 2 mM EDTA, pH 7.6). The lysed cells were centrifuged at 14000 rpm for 10 min at 4 °C. The amount of Cy3-labeled ODN in the supernatant was determined by examining its fluorescence intensity on a LS50B Perkin-Elmer Luminescence Spectrometer. The protein content of the supernatant was measured with Bio-Rad protein assay system (Bio-Rad, Hercules, CA). The result of ODN uptake was expressed as the pg Cy3-labeled ODN/μg protein.

Fluorescence Microscopic Examination of Cellular Uptake of Folate-Targeted Lipidic ODN. KB cells were seeded at a density of 1×10^5 cells/well in a 4-well Nalge Nunc Lab-Tek chamber slide (Naperville, IL) and kept overnight at 37 °C. ODN, free or formulated in lipid vesicles with or without a folate ligand, were added at a Cy3-ODN concentration of 100 ng/mL. In one well, free folate was added to the folate-targeted lipidic ODN at a final concentration of 1 mM. One hour following the incubation at 37 °C cells were washed three times with PBS. Cells were then fixed with 2% paraformaldehyde at 37 °C for 20 min and further washed three times with PBS. Cellular uptake of Cy3-labeled ODN was examined under a Nikon Eclipse TE 300 fluorescence microscope with a blue filter at 200× magnification.

RESULTS

Preparation and Characterization of Folate-Targeted, ODN-Containing Lipid Vesicles. Cy3-labeled ODN were used as a label for quantification of ODN encapsulation efficiency and for following the cellular uptake of ODN-containing lipid vesicles. Cy3-labeled ODN were mixed with unlabeled ODN at a 1:9 ratio (w/w). ODN were first mixed with a lipid solution composed of DSPC, cholesterol, DODAP, PEG-CerC_{16}, and folate PEG DSPE under an acidic condition in the presence of 40% ethanol. DODAP/ODN complexes and other lipids were then self-assembled to form ODN-containing lipid vesicles during the dialysis against 300 mM citrate buffer, pH 4.0, and then against HBS (20 mM HEPES, 145 mM NaCl, pH 7.6). Figure 1 shows the profile of separation of the mixture on Sepharose CL-4B. The lipid-associated ODN were well separated from free ODN. The size of the ODN-containing vesicles ranges from 100 to 200 nm with 60 80% of input ODN being associated with the lipid vectors. These vesicles carry a negative charge surface as confirmed by Zeta potential analysis (data not shown). The ODN that were associated with lipid vesicles were fully protected from degradation by nuclease, suggesting that ODN were encapsulated inside the lipid vesicles (data not shown). These studies were performed with six different ODN ranging from 15 to 23 mer in length (Table 1), and similar results were obtained.

Effect of the Amount of Folate PEG DSPE on the Cellular Uptake of Folate-Targeted Lipidic ODN. Figure 2 shows the cellular uptake of lipidic ODN by KB cells with increasing amount of input folate PEG DSPE. The total amount of lipid-derivatized PEG (folate PEG DSPE plus PEG-CerC_{16}) added is 2 mol %. Increasing the amount of folate from 0.1 to 0.5 mol % resulted in a significant increase in the cellular uptake of ODN. Increasing the input folate beyond 0.5 mol % was not associated with a further increase in the cellular uptake of ODN.

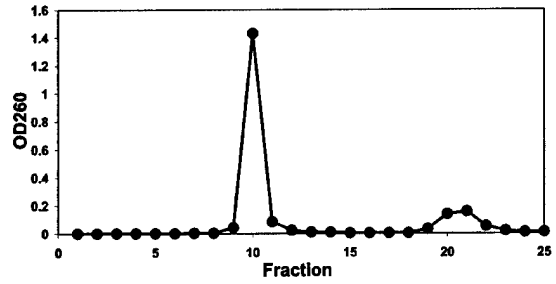

Figure 1. Size-exclusion chromatographic fractionation of ODN-containing lipid vesicles on a Sepharose CL-4B column. DSPC, cholesterol, DODAP, PEG-CerC$_{16}$, and folate PEG DSPE were individually dissolved in ethanol and mixed in a molar ratio of 25/45/25/4/1. To the lipid solution was added 300 mM citric acid, pH 4.0, to a final ethanol concentration of 40% (v/v). Similarly ODN were prepared in separate tubes in 300 mM citric acid, pH 4.0, with 40% ethanol. The solutions were prewarmed to 65 °C, and the lipids were slowly added to the ODN with gentle vortexing. The mixture was passed 10 times through three stacked 100 nm polycarbonate filters. The preparation was dialyzed (12 14 kDa cutoff) against 300 mM citrate buffer, pH 4.0, for approximately 1 h to remove excess ethanol and further dialyzed against HBS (20 mM HEPES, 145 mM NaCl, pH 7.6) for 12 18 h. The dialyzed solution (0.5 mL) was loaded on to a Sepharose CL-4B column (1 cm × 25 cm), which had been equilibrated with HEPES (pH7.6). Lipidic ODN and free ODN were collected with 1 mL in each fraction and examined for their absorbance at 260 nm.

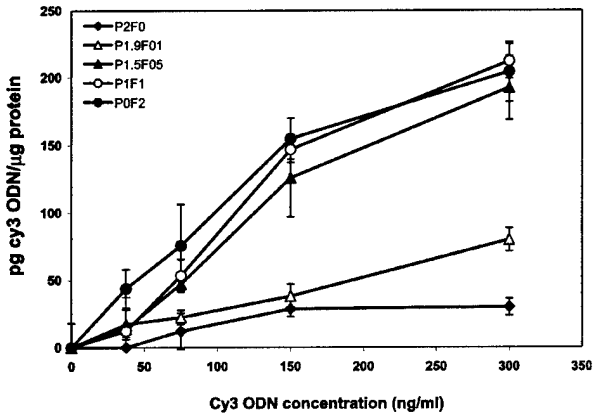

Figure 2. Effect of the amount of folate-PEG-DSPE on the cellular uptake of folate-targeted lipidic ODN. ODN-containing lipid vesicles were prepared as described in the legend to Figure 1 with various amounts of input folate PEG DSPE. The total amount of PEG added in the lipid mixture is 2 mol %. KB cells were seeded to a 24-well plate at a cell density of 2.5 × 10^4/well and allowed to grow overnight. Lipidic ODN were added to the cells in various concentrations (determined according to the Cy3-labeled ODN). Following incubation at 37 °C for 1 h, cells were washed with PBS and then lysed using a lysis buffer. The lysed cells were centrifuged at 14000*g* for 10 min at 4 °C. The amount of Cy3-labeled ODN in the supernatant was determined by examining its fluorescence intensity on a fluorometer. The protein content of the supernatant was measured with Bio-Rad protein assay system. The result of ODN uptake was expressed as the pg Cy3-labeled ODN/μg protein. The *x* and *y* in PxFy (Figures 2, 3, and 5) represent the mol % of PEG-Cer16 and folate PEG DSPE in the lipid vesicles, respectively. *n* 3.

Effect of the Amount of Ceramide-Derivatized PEG on the Cellular Uptake of Folate-Targeted Lipidic ODN. Figure 3 shows the effect of the amount of input ceramide-derivatized PEG on the cellular uptake of folate-targeted lipidic ODN. The amount of input folate PEG DSPE is 0.1 (Panel A) or 1 mol % (Panel B). Increasing the amount of ceramide-derivatized PEG from 0 to 10 mol % resulted in a significant decrease in

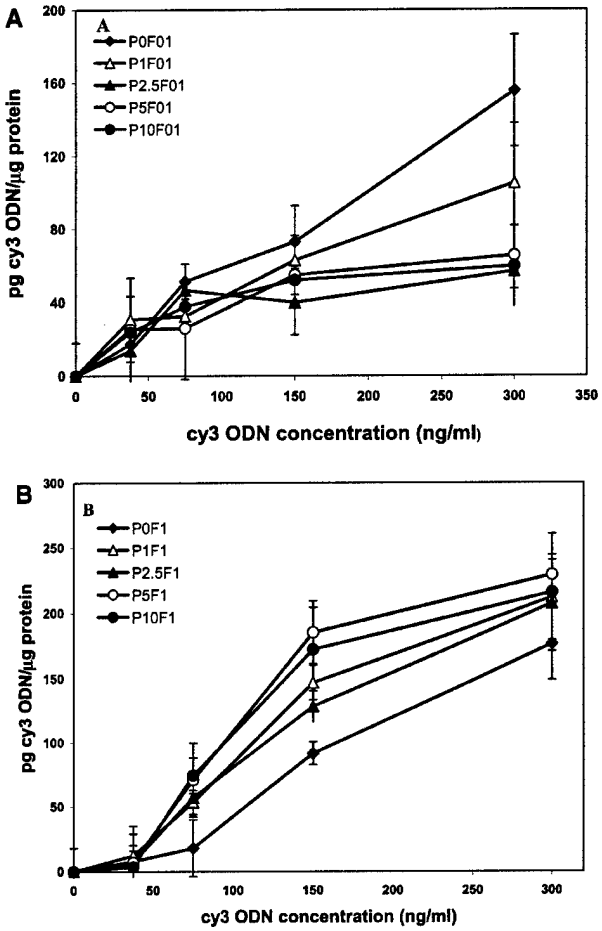

Figure 3. Effect of the amount of ceramide-PEG on the cellular uptake of folate-targeted lipidic ODN. ODN-containing lipid vesicles were prepared as described in the legend to Figure 1 with various amounts of input ceramide-PEG. The amount of input folate PEG DSPE was 0.1 mol % (Panel A) or 1 mol % (Panel B). Various amounts of ODN were added to KB cells and the cellular uptake of ODN was evaluated as described in the legend to Figure 2. *n* 3.

the cellular uptake of ODN when 0.1 mol % of folate PEG DSPE was used (Panel A). However, such an inhibitory effect was diminished when the amount of input folate PEG DSPE was increased to 1 mol % (Panel B).

Effect of PEG Length on the Cellular Uptake of Lipidic ODN. Previous studies have shown that folate-mediated targeting of liposomes is affected by the length of PEG spacer between folate and the lipid anchor (such as DSPE in this study) (*10, 11*). Similar results were observed in this study with ODN targeting using our new lipidic vector (Figure 4). Folate with a PEG of 3500 Da appeared to be more efficient than that with a PEG of 2000 Da in mediating the targeting of ODN to KB cells. This difference became more dramatic with increasing amounts of ceramide PEG (data not shown).

Cellular Uptake of Folate-Conjugated, Lipidic ODN is Mediated by Folate Receptors. To demonstrate whether the cellular uptake of folate-conjugated, lipidic ODN is mediated by folate receptor, their uptake was also examined on CHO cells, which have a low level of folate receptors (Figure 5). As shown in Panel A, coupling of folate to lipidic ODN led to a significant increase in the cellular uptake by KB cells. The level of ODN uptake was about 8 10-fold higher for folate-

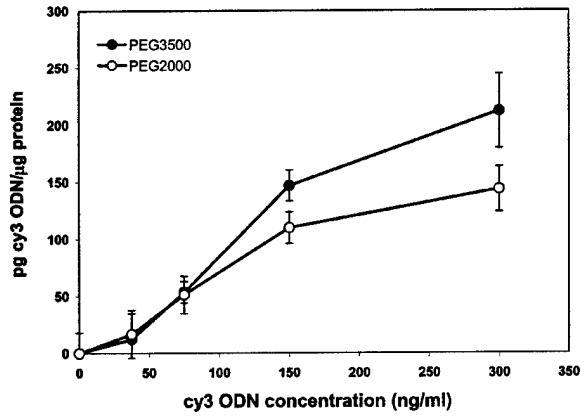

Figure 4. Effect of PEG length on the cellular uptake of lipidic ODN. Lipidic ODN were prepared as described in the legend to Figure 1. The molecular weight of PEG in folate PEG DSPE was 2000 or 3500 Da. Various amounts of ODN were added to KB cells and the cellular uptake of ODN was evaluated as described in the legend to Figure 2. *n* 3.

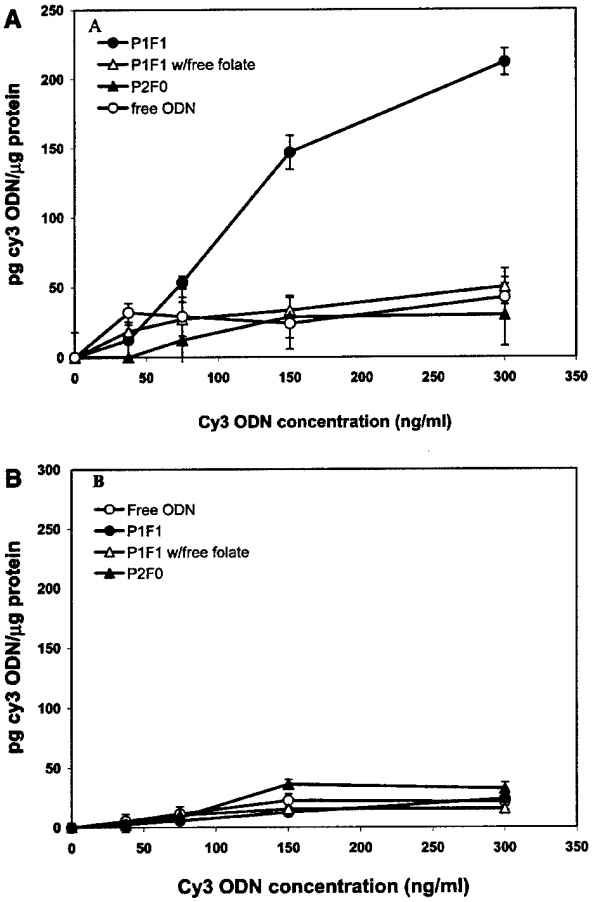

Figure 5. Cell type-specific uptake of folate-targeted lipidic ODN. KB (Panel A) or CHO (Panel B) cells were plated in a 24-well plate. ODN, free or formulated in lipid vesicles with or without a folate ligand, were added to cells in various concentrations. In a separate experiment, free folate was added to folate-targeted lipidic ODN at a final concentration of 1 mM. Cellular uptake of ODN was then evaluated as described in the legend to Figure 2. *n* 3.

targeted lipidic ODN than that of free ODN or lipidic ODN without a ligand. The improvement in ODN uptake was almost completely blocked by adding excess amount of free folate. There was essentially no difference in the ODN uptake by CHO cells whether ODN were free or formulated in ligand-free lipid vesicles or folate-targeted lipid vesicles (Panel B).

Fluorescence Microscopic Examination of the Cellular Uptake of Folate-Targeted Lipidic ODN. Figure 6 shows the fluorescence images of ODN uptake by KB cells. At 1 h following incubation at 37 °C, folate-targeted ODN were found to be associated with the cell membrane as well as inside the cells (Panel B). In agreement with the quantitative analysis (Figure 5), the level of cell-associated fluorescence intensity is substantially higher with folate-targeted ODN (Panel B) than that with either free ODN (Panel D) or ODN formulated in nontargeted lipid vesicles (Panel A). However, the fluorescence intensity with folate-targeted ODN was decreased to background level by adding excess amounts of free folate (Panel C). These results suggest that the binding and the subsequent internalization of the folate-targeted ODN are mediated by the folate receptors.

Effect of Serum on ODN Uptake by KB Cells. Figure 7 shows the uptake of folate-targeted ODN by KB cells in the presence or absence of 10% FBS. Serum appears to have only a slight effect on the cellular uptake of folate-targeted ODN. Similar phenomenon was observed for free ODN or ODN formulated in nontargeted lipid vesicles (data not shown).

DISCUSSION

An ideal ODN vector should have a large ODN loading capacity and deliver the ODN to target cells in a cell-type-specific manner. Neutral liposomes are suitable for active targeting but suffer from low encapsulation efficiencies and drug-to-lipid ratios. This problem can be resolved via incorporation of cationic lipids into the formulation, but the resulting lipid/nucleic acid complexes are generally unstable and short-lived in blood circulation. Furthermore, substantial amounts of ODN are associated with the positively charged surface of the particles. These ODN may be released from the particles by the highly negatively charged molecules in the blood following systemic administration (*12*). Recently, Stuart and Allen (*13*) described a method by which 80 100% of input ODN were entrapped in a lipid vector that was stable in human plasma. This involves the formation in and extraction of cationic lipid/ODN complex from an organic solvent. The cationic lipid/ODN complex was then mixed with other neutral lipids and ODN-containing lipid vesicles were obtained by a reverse phase evaporation method (*13*). More recently, a much simpler method was developed by Semple and colleagues (*1*), which utilizes an ionizable aminolipid (DODAP) and an ethanol-containing buffer system. A similar level of encapsulation efficiency was achieved (*1*). These ODN-containing lipid vesicles, however, are inefficient in interacting with cells. Furthermore, they lack cell type specificity. In this study we investigated whether a targeting ligand can be incorporated into these vesicles to further improve their efficiency in intracellular delivery of ODN. Folate was chosen as a targeting ligand since folate receptors have been shown to be overexpressed in a number of cancer cells. Folate has been used successfully in targeting to tumors various types of agents including chemotherapeutic drugs (*10*), radionucleotides (*14, 15*), ODN (*6, 16*), DNA (*17, 18*), etc. The advantages of a folate-targeting system include its excellent safety profile and the low immunogenicity. Folate receptors have also been shown to mediate efficient internalization of free folate or the

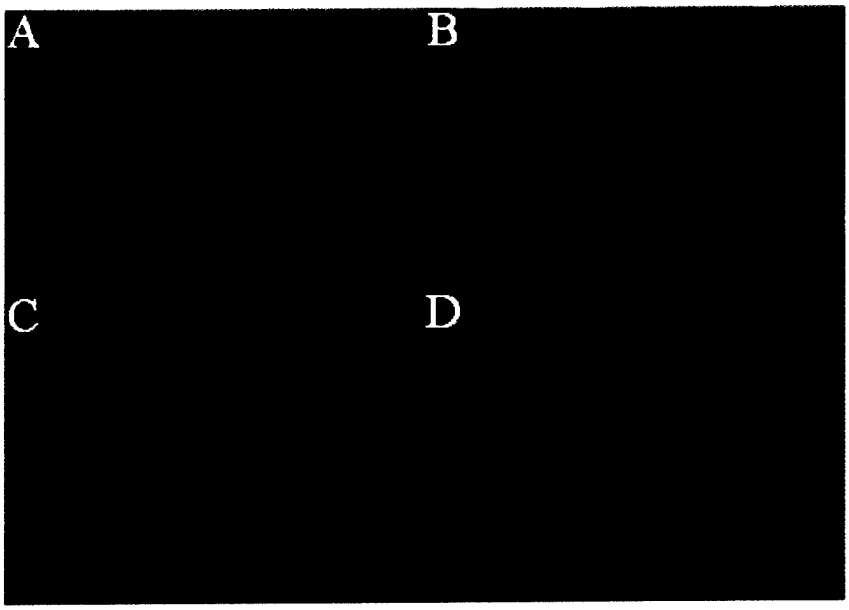

Figure 6. Fluorescence microscopic examination of cellular uptake of folate-targeted lipidic ODN. KB cells were seeded at a density of 1×10^5 cells/well in a 4-well Nalge Nunc Lab-Tek chamber slide (Naperville, IL) and kept overnight at 37 °C. ODN, free or formulated in lipid vesicles with or without a folate ligand, were added at a Cy3-ODN concentration of 100 ng/mL. In one well, free folate was added to the folate-targeted lipidic ODN at a final concentration of 1 mM. One hour following the incubation at 37 °C cells were washed three times with PBS. Cells were then fixed with 2% paraformaldehyde at 37 °C for 20 min and further washed with PBS three times. Cellular uptake of Cy3-labeled ODN was examined under a Nikon fluorescence microscope with a blue filter at 200X magnification. A: Lipidic ODN without a ligand; B: folate-targeted lipidic ODN; C: folate-targeted lipidic ODN + 1 mM free folate; D: free ODN.

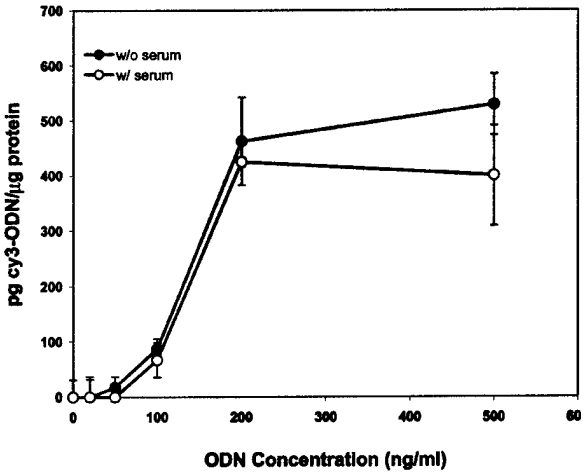

Figure 7. Effect of serum on ODN uptake by KB cells. KB cells were seeded to a 24-well plate at a cell density of 2.5×10^4/well and allowed to grow overnight. Various concentrations of folate-targeted ODN were added to cells in the presence or absence of 10% FBS. Cellular uptake of ODN was then evaluated as described in the legend to Figure 2. $n = 3$.

conjugates, which should facilitate intracellular delivery of the therapeutic agent. Results from this study clearly show that incorporation of folate into the ODN-containing vesicles leads to a significant improvement in delivery of ODN to KB cells that overexpress folate receptors (Figures 5 and 6). The increase in the ODN uptake was almost completely blocked by excess amount of free folate (Figures 5 and 6). Conjugation of folate led to little change in ODN uptake by CHO cells that are negative for folate receptor expression (Figure 5). These studies clearly indicate that targeted delivery of ODN to KB cells via this novel lipid vector is mediated by the folate receptors.

The targeting efficiency of folate-conjugated vesicles is affected by the amount of input folate–PEG–DSPE. Increasing the amount of folate from 0 to 0.5 mol % was associated with a significant increase in ODN uptake. However, increasing the input folate beyond 0.5 mol % was not associated with a further increase in the cellular uptake of ODN (Figure 2). Interestingly, the targeting efficiency of folate-conjugated vesicles is also affected by the amount of input ceramide–PEG (Figure 3). Ceramide–PEG was included for two purposes: (a) to render the resulting particles long-circulating in the blood, and (b) to prevent aggregation during the preparation of ODN-containing lipid vesicles, particularly when the vectors were prepared at high lipid concentrations. However, at a high concentration of ceramide-PEG, substantial amounts of PEG have been shown to be excluded from the lipid vesicles (*1*). Similarly, some of the input folate might be excluded from the vesicles, which may lead to incoporation of less than sufficient amounts of folate needed to achieve efficient targeting when a small amount of input folate–PEG–DSPE is used. This may explain the fact that, at a folate concentration of 0.1 mol %, the efficiency of ODN delivery was decreased dramatically when the ceramide–PEG was increased to above 2.5 mol % (Figure 3A). This problem, however, can be resolved by increasing the amount of input folate–PEG–DSPE. As shown in Figure 3B, increasing the amount of input folate from 0.1 to 1 mol % resulted in a full recovery of targeting efficiency at high concentrations of ceramide–PEG (above 5 mol %). No purification is required to remove the free folate–PEG–DSPE from the folate-conjugated, ODN-containing lipid vesicles.

Similar to liposomal targeting (*10*, *11*), the efficiency of ODN delivery via the new lipid vector is affected by the length of the PEG spacer. This might be due to the fact that the PEG coating imposes steric hindrance for

the folate to interact efficiently with the folate receptors on the cell surface when a short PEG spacer (such as 2000 Da) is used. This interference becomes more pronounced with increasing amounts of ceramide PEG (data not shown). This problem, however, can be resolved via the use of a longer PEG spacer between the folate and the lipid anchor. As shown in Figure 4, increasing the length of PEG spacer from 2000 to 3500 Da results in a significant improvement in the targeting efficiency. Furthermore, the targeting efficiency of the resulting lipid vectors is not significantly affected by the amount of the input ceramide PEG (Figure 3B).

In summary, we have developed a novel lipid vector that is highly efficient in delivering ODN to tumor cells that overexpress folate receptor. We have also shown recently that this vector mediates efficient delivery of ODN to mouse lung endothelial cells using an endothelial cell-specific antibody as a ligand (Wilson et al., unpublished data). Currently we are examining the targeting efficiency of this vector in vivo.

ACKNOWLEDGMENT

This work was supported by DOD grant PC001525 and NIH grant HL RO1 63080 (to S Li). We would like to thank Dr. Takuro Niidome for his helpful discussion in this study.

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BC025569Z